Brief Definitive Report

Pgs28 Belongs to a Family of Epidermal Growth Factor–like Antigens That Are Targets of Malaria Transmission–blocking Antibodies

By Patrick E. Duffy,*§ Paulo Pimenta,‡ and David C. Kaslow*

*From the *Molecular Vaccine Section, Laboratory of Malaria Research, and the ‡Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892; and the §Department of Medicine, Walter Reed Army Institute of Research, Washington, DC 20307*

Summary

Although Pgs28, a 28-kD surface protein of *Plasmodium gallinaceum* oökinetes, was previously thought not to be a target of transmission-blocking antibodies, we found that polyclonal antisera to Pgs28 completely blocked parasite infectivity to *Aedes aegypti* mosquitoes. Antisera raised against reduced Pgs28 were less effective in blocking transmission than were antisera to nonreduced Pgs28; thus, the target epitope(s) of transmission-blocking antibodies appears to be conformation dependent. In stage-specific assays, polyclonal antisera impaired the in vitro transformation of zygotes to mature oökinetes, as well as the in vivo development of mature oökinetes to oöcysts. Using microsequence of immunoaffinity-purified Pgs28, we cloned the 666-bp open reading frame of the Pgs28 gene. The deduced amino acid sequence of Pgs28 is strikingly similar to that of a *P. gallinaceum* zygote surface protein, Pgs25, and its *P. falciparum* analogue, Pfs25. Pgs28, like Pgs25 and Pfs25, has a presumptive secretory signal sequence, followed by four epidermal growth factor–like domains, and a terminal hydrophobic region.

Antibodies to surface proteins of sexual stage malarial parasites can completely inhibit infectivity to the mosquito vector, and thus block transmission of malaria (1–4). A series of antigens, identified as targets of transmission-blocking antibodies, sequentially appear on the surface of the parasite as it develops in the mosquito midgut (3, 5, 6). Because drug-resistant parasite strains and insecticide-resistant mosquitoes have rendered current control measures increasingly ineffective, several of these sexual stage antigens have become attractive candidates for a transmission-blocking vaccine to control the spread of malaria (7). A combination of these transmission-blocking target antigens in a "cocktail" vaccine may elicit a more efficacious immune response of greater duration and have broader immunogenicity than a transmission-blocking vaccine that consists of just a single antigen.

Plasmodium gallinaceum is a facile model for the study of transmission-blocking vaccines and sexual stage antigens because of its unique capability to undergo complete sporogonic development in vitro (8) and because of its phylogenetic proximity to *P. falciparum* (9, 10). In particular, the expression of surface proteins during the sexual stages of their life cycle is strikingly similar in *P. gallinaceum* and *P. falciparum* (6). 8 yr ago, Grotendorst et al. (11) described a 28-kD *P. gallinaceum* surface protein, Pgs28, that is immunoprecipitated from extracts of zygotes/oökinetes by mAbs that suppress but do not block malaria transmission. Based on recent evidence that polyclonal sera to Pfs25, a 25-kD transmission-blocking target antigen present on the surface of *P. falciparum* zygotes, are qualitatively better than mAbs in blocking transmission (12), we evaluated whether polyclonal sera to Pgs28 might be more potent blocking reagents than the mAbs studied previously. Here we report that murine polyclonal sera against immunoaffinity-purified Pgs28 completely block parasite transmission. We also describe the deduced amino acid sequence of Pgs28, which places it in a family of sexual stage antigens with epidermal growth factor (EGF)-like domains.

Materials and Methods

Oökinete Antigens. Purified *P. gallinaceum* (isolate 8A) zygotes were prepared from parasitized blood of infected White Leghorn chickens and transformed in vitro into oökinetes as previously described (13). Antigens were extracted with 50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.02% NaN_3, pH 7.4.

Purification of Pgs28. Pgs28 was immunoaffinity purified from oökinete extracts using mAb IID2-B3B3 (11) covalently linked to protein A–Sepharose 4B beads (ImmunoPure® Kit; Pierce Chemical Co., Rockford, IL), eluted from the resin by electrophoresis in 50 mM NH_4HCO_3, 0.1% SDS for 4 h at 10 mA (14), and size fractionated by SDS-PAGE (10% polyacrylamide gel) in nonreducing or reducing conditions. Approximately 5 μg of Pgs28 was elec-

troblotted from the gel onto pure nitrocellulose, in situ digested with trypsin (15), and microsequenced (William Lane, Harvard MicroChemistry, Cambridge, MA), and ~1 μg was electroblotted onto polyvinylidene difluoride for NH_2-terminal sequence (Dr. John Coligan, Biological Resources Branch, National Institutes of Health, Bethesda, MD).

Screening Genomic DNA Library. Tryptic peptide sequences of Pgs28 were used to construct synthetic degenerate oligonucleotide probes. A genomic library of HindIII-digested *P. gallinaceum* DNA was constructed in pUC13. Filter-immobilized *Escherichia coli* colonies were screened with 12 ng of radiolabeled oligonucleotide probe NT14AGT: 5′-TT (AG)TT (AG)TC (TC)TT GTA TGG (AG)TC (TC)TC-3′, by hybridization at 45°C for 16 h. The filters were washed at a final stringency of 6× SSC (1 M sodium chloride, 0.1 M sodium citrate, pH 7.0), 0.1% SDS at 49°C for 5 min (16). Autoradiography at −70°C for 4–16 h was performed to identify positive colonies. The complete sequence of the open reading frame from both strands of clone 9A1 was determined by the dideoxynucleotide terminator method.

Nucleic Acid Blotting. For Northern blots, total cellular RNA from 5 × 10^7 5-h-old *P. gallinaceum* zygotes was size fractionated per lane on a 0.8% agarose-formaldehyde gel and blotted by capillary transfer to a nylon membrane (Nytran; Schleicher & Schuell, Inc., Keene, NH) (17). For Southern blots, 0.5 μg of genomic DNA from *P. gallinaceum* parasites was digested by one or more restriction endonucleases, subjected to electrophoresis through a 1% agarose gel, and transferred to a nylon membrane (18). Filters were hybridized overnight at melting temperature (T_m) − 10°C with ^{32}P-labeled probes, then washed with 6× SSC, 0.1% SDS at T_m − 5°C for 5 min (Southern blots) or 7 min (Northern blots).

Immunizations. Immunoaffinity-purified, SDS-PAGE size-fractionated Pgs28 in polyacrylamide gel was emulsified in Monophosphoryl Lipid A + Trehalose Dimycolate (MPL® + TDM), according to the manufacturer's protocol (Ribi ImmunoChem Research, Inc., Hamilton, MN). Male BALB/c mice, aged 4–6 wk, were immunized up to seven times intraperitoneally with 0.2 ml of the emulsion (~1 μg of protein). The control group of mice received polyacrylamide gel, without antigen, emulsified in MPL® + TDM adjuvant.

Immunoelectromicroscopy. Mature oökinetes were prepared for electron microscopy by standard techniques (19) using mAb IID2-B3B3 and goat anti-mouse antibodies (EY Labs, Inc., San Mateo, CA) conjugated with colloidal gold (10–15 nm).

Transmission-blocking Assays. The method of quantifying transmission-blocking antibodies in vitro has been previously described (11). Briefly, mosquitoes were fed test antibodies mixed with *P. gallinaceum*–parasitized material (either infected chicken blood or mature oökinetes mixed with naive chicken blood) through an artificial membrane feeding apparatus. Infectivity was measured 1 wk after the infectious bloodmeal by counting the number of oöcysts per mosquito midgut of ~20 mosquitoes. Two endpoints of transmission-blocking activity in test sera were analyzed: the percentage of mosquitoes in a batch that had one or more oöcysts on their midgut, and the number of oöcysts per midgut. Mosquito batches fed on immune sera were compared with those fed on controls. The percentage of mosquitoes with oöcysts was compared by χ^2 analysis. The number of oöcysts/midgut was compared by Wilcoxon's rank sum analysis.

Results and Discussion

Pgs28 Is a Major Surface Protein of P. gallinaceum Oökinetes Distinct from Pgs25. Immunoelectron microscopy, using mAb IID2 B3B3, demonstrated the uniform and extensive distri-

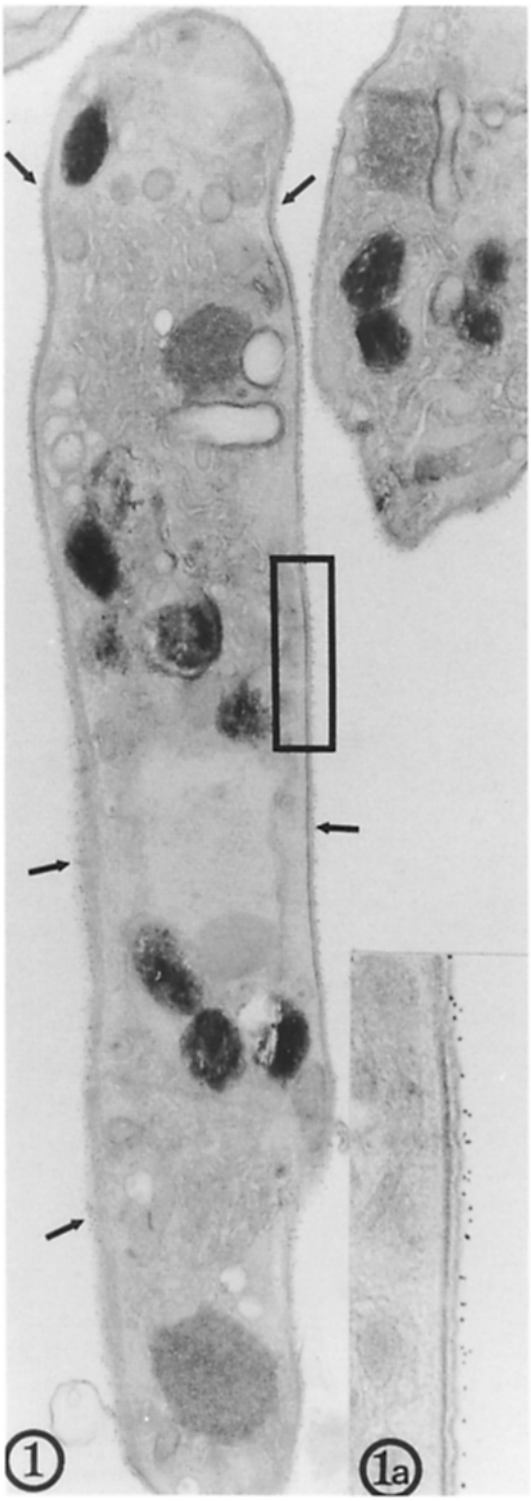

Figure 1. Distribution of Pgs28 on the surface of mature oökinetes. Immunogold labeling of Pgs28 with mAb IID2-B3B3 showed extensive distribution of gold particles over the entire cell surface of a whole mature oökinete (×21,250). Detail of the cell membrane (*a*, ×59,250) showed gold particles above the cell coat.

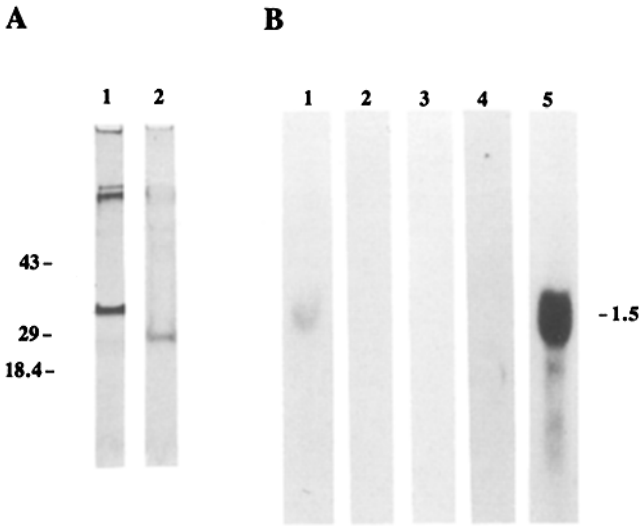

Figure 2. SDS-PAGE and Northern blot analysis of Pgs28. (*A*) Non-reduced (lane *1*) or reduced (lane *2*) immunoaffinity-purified Pgs28 was size fractionated by a 10% SDS-polyacrylamide gel and stained with coomassie blue. mAb IID2-B3B3 recognized the dominant band at 34 kD (lane *1*) by Western blot, but failed to recognize a band in the reduced material (Western blot data not shown). On the left is molecular mass in kilodaltons. (*B*) Northern blot analysis of total RNA obtained from 5-h-old *P. gallinaceum* zygotes hybridized with a 23-mer oligonucleotide, either completely degenerate at nucleotide position no. 15 (lane *1*), or replaced with adenosine (lane *2*), cytosine (lane *3*), guanosine (lane *4*), or thymidine (lane *5*) only at that final position. On the right is molecular weight in kilobases.

bution of Pgs28 on the surface of the mature oökinete (Fig. 1), corroborating earlier biosynthetic work that showed that Pgs28 is the predominant surface protein of mature oökinetes (5). Thus, Pgs28 is distinct from Pgs25, which achieves peak synthesis in the early hours after parasite fertilization (5). Pgs28 and Pgs25 can also be differentiated by their apparent M_r on SDS-PAGE, as well as their specific recognition by mAbs (11). By Western blot analysis (data not shown), Triton X-100 extracts of oökinetes depleted of Pgs28 by chromatography with mAb IID2-B3B3 (specific for Pgs28) were not depleted of Pgs25 as assayed by mAb IID2-C5I (specific for Pgs25). Furthermore, by Western blot analysis the immunoaffinity-purified Pgs28 (Fig. 2) was recognized by mAb IID2 B3B3 but not by mAb IID2-C5I (data not shown).

Polyclonal Sera Directed against Pgs28 Completely Block Malaria Parasite Transmission and Act at More than One Stage in the Life Cycle. mAbs to Pgs28 were previously shown to suppress the development of oöcysts after an infectious bloodmeal, decreasing infectivity (measured as geometric mean number

Table 1. *Transmission-blocking Activity of Sera from Immunized Animals*

Sample	Mean oocyst number (range)	Infectivity (percent of control)	Mosq. infected/ mosq. dissected
Preimmune	39.2 (21–62)		4/4
Anti-Pgs28 (IID7)	0 (0)	0	0/5
Control (IID8)*	5.3 (0–30)		22/33
Anti-Pgs28 (IID8)*	0.02 (0–1)	<0.01	1/41
Anti-red.Pgs28 (IID8)*	3.2 (0–32)	60.4	8/30
Control (IID13)	10.5 (0–37)		10/15
Anti-Pgs28 (IID13)	0 (0)	0	0/19
Control (VIID7)	13.0 (5–26)		6/6
Anti-Pgs28 (VIID7)	0 (0)	0	0/20
Control (IID14)	5.0 (0–41)		4/10
Anti-Pgs28 (IID8)	0 (0)	0	0/12
Control (IID8)	33.5 (0–302)		17/20
Anti-Pgs28 (IID8)	4.2 (0–56)	12.5	7/21
Anti-red.Pgs28 (IID8)	6.6 (0–28)	29.8	9/14

Pooled sera from two to three BALB/c mice either before immunization (Preimmune), after immunization with adjuvant alone (Control), or with nonreduced or reduced immunoaffinity-purified Pgs28 in adjuvant (Anti-Pgs28 or Anti-red.Pgs28, respectively) were mixed with parasite-infected chicken blood. Mosquitoes were dissected after 6–8 d, and the numbers of infected mosquitoes and oocysts determined as previously described (11). Each sample is identified by immunization number (Roman numeral) and the number of days (D) postimmunization (Arabic numerals). Shown from five separate experiments is the infectivity calculated as the mean oocyst count of the test group divided by the mean oocyst count of the control group fed at the same time (× 100). On the bottom, the ability of sera to inhibit in vivo development of ookinetes into oocysts was measured (11). The transmission blocking assay was performed as above, except that in vitro cultured ookinetes were used rather than parasite-infected chicken blood.
* Combined results from two separate experiments.

Table 2. *In Vitro Transformation-blocking Activity of Sera from Immunized Animals*

Sample	No. of ookinetes	Total no. of parasites	Percent transformation
Control	156	373	41.8
Anti-Pgs28	31	368	8.4

Sera from mice immunized with adjuvant or Pgs28 (see Table 1) were diluted 1:8 to 1:10 in M199 media containing 3–5 × 10^6 purified *P. gallinaceum* zygotes. The parasites were then allowed to transform into mature oökinetes overnight. Only completely elongated forms, and not retorts or round forms, were counted as oökinetes. Total number of parasites includes only sexual stage parasites; the rarely observed asexual stage parasite or chicken leukocyte was not included. The combined results of three separate experiments are shown. The differences observed in the percent transformation were statistically significant ($p < 0.05$) in each of three separate experiments by χ^2 analysis.

oöcysts/midgut) to 38–48% of control (11). In contrast, we found that after a single boost with immunoaffinity-purified Pgs28, BALB/c mice generated polyclonal, monospecific antisera with complete transmission-blocking activity. In fact, in five transmission-blocking assays, 85 mosquitoes received Pgs28 antisera, of which only a single mosquito was infected, and in that mosquito only a single oöcyst developed (Table 1).

Polyclonal antisera against Pgs28 impair at least two distinct stages of parasite sexual development: (*a*) the transformation of zygotes to oökinetes; and (*b*) the development of oökinetes to oöcysts. During an overnight incubation in M199, *P. gallinaceum* zygotes readily transform into elongated oökinetes. The addition of Pgs28 antisera to the culture media significantly reduced the proportion of parasites that underwent this in vitro transformation (Table 2). In vivo, oökinetes traverse the midgut epithelium, then lodge beneath the basal lamina to develop into oöcysts. This development occurs even when mosquitoes are fed mature oökinetes grown in vitro; however, the proportion of mosquitoes that develops oöcysts was significantly reduced when Pgs28 antisera rather than control sera were added to a bloodmeal containing mature oökinetes (Table 1). The incubation of mature oökinetes with Pgs28 antisera in vitro did not induce parasite death (data not shown). The clear superiority of polyclonal antibodies over mAbs against Pgs28 and against Pfs25 (12) may represent the combined result of multiple blocks in parasite development. These data also emphasize the limitations of using the now classical mAb approach to identify transmission-blocking target antigens.

Oligonucleotide Probes Used to Clone the Gene Encoding Pgs28 Were Deduced from the NH_2-terminal Amino Acid Sequence of

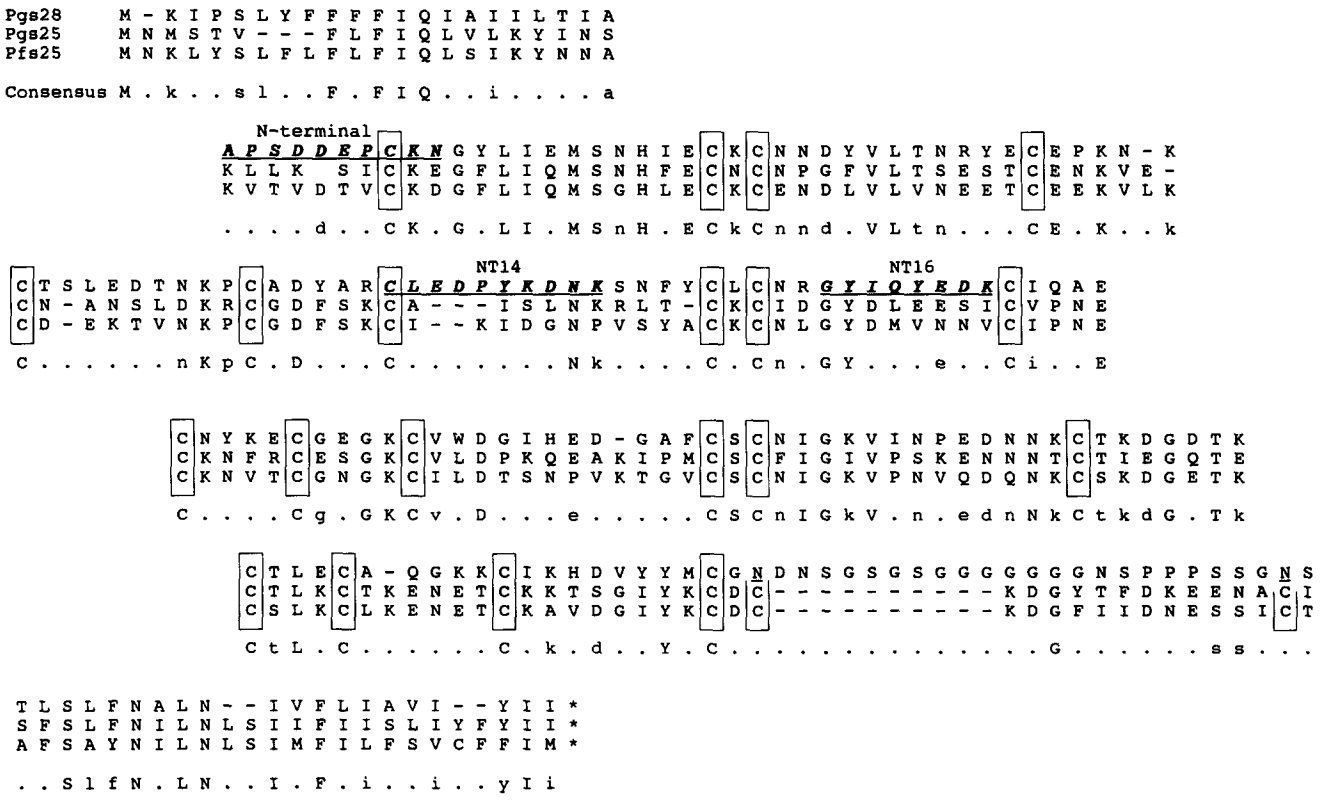

Figure 3. Deduced amino acid sequence of Pgs28 compared with that of Pgs25 and cPFs25. The sequences obtained from the NH_2 terminus of the mature protein and from two tryptic peptides (NT14 and NT16) are shown as underlined, bold italics. The consensus sequence shown as uppercase letters is for those amino acid residues identical in Pgs28, Pgs25 (19), and Pfs25 (15), and as lowercase letters for those residues identical in Pgs28, and Pgs25 or Pfs25. The cysteine residues present in EGF-like domains are enclosed in boxes.

Tryptic Peptides from Pgs28. Immunoaffinity purification of Pgs28 from crude oökinete extract resulted in a predominant band of 34 kD on 10% SDS-PAGE (Fig. 2 *A*), which was electroblotted onto polyvinylidene difluoride for NH_2-terminal sequencing (Fig. 3). Reduction of disulfide bonds by treatment of the immunoaffinity-purified material with β-mercaptoethanol caused Pgs28 to comigrate on SDS-PAGE with the small amount of mouse light chain that coeluted from the immunoaffinity column (Fig. 2 *A*). After blotting onto nitrocellulose membrane, the reduced protein was digested with trypsin, and eluted peptides were separated by reverse-phase HPLC. Three tryptic peptides were sequenced, of which two (NT14 and NT16; Fig. 3) were unique when screened in Swiss Prot (Release 17; Centre Medicale Universitaire, Geneva, Switzerland), and one was identical with mouse antibody light chain.

Completely degenerate oligonucleotide probes recognized a 1.5-kb transcript by Northern blot hybridization with total RNA from 5-h-old zygotes (Fig. 2 *B*), but failed to detect the gene by either Southern blot hybridization with genomic digests or colony screening of existing cDNA and genomic libraries (data not shown). A greatly enhanced signal by Northern blot analysis was obtained with guanosine at position 12 and thymidine at position 15 (Fig. 2 *B*). This probe (NT14AGT) identified a 3.3-kb band on Southern blot hybridization of a HindIII digest of *P. gallinaceum* genomic DNA (data not shown), and subsequently identified a positive clone (p9A1) in a library of HindIII-digested genomic DNA ligated into pUC13 (data not shown).

The Deduced Amino Acid Sequence of Pgs28 Demonstrates Significant Homology with Pgs25 and Pfs25. The 666-bp open reading frame (GenBank accession no. M96886) within p9A1 yielded a deduced amino acid sequence that contained all three of the Pgs28 peptides that were microsequenced (Fig. 3). The only misread occurred at deduced amino acid residue position 28 (sequenced as proline in the eighth position of the mature protein; deduced as cysteine 28 in p9A1) (Fig. 3). The homology between Pgs28 and both Pgs25 and Pfs25 (20) is striking: all three proteins contain a putative secretory signal sequence, then four EGF-like domains, and finally a short, COOH-terminal hydrophobic region without a hydrophilic (cytoplasmic) tail. Even though attempts to cleave these proteins from the surface of sexual stage parasites with phosphatidylinositol-specific phospholipase C (PI-PLC) have not yet been successful (data not shown), the short hydrophobic COOH terminus, along with previous data indicating that these proteins incorporate the appropriate lipids and glycans (5), strongly suggest that all three proteins are anchored to the cell membrane of the parasite by glycosylphosphatidylinositol (GPI).

The six-cysteine motif of EGF-like domains shared between these proteins does not necessarily imply a shared function, as these domains have been recognized in a number of proteins with diverse functions (21), including transmembrane, extracellular matrix, and soluble, secreted proteins. For example, $MSP1_{19}$, the 19-kD surface polypeptide on *P. falciparum* merozoites/rings, is a malarial parasite antigen with EGF-like domains (22). As MSP1 is expressed solely in asexual stage parasites, its function is likely to be different than those of the sexual stage–specific proteins, Pgs25 (and the *P. falciparum* analogue, Pfs25) and Pgs28.

Besides structural and biochemical characteristics, Pgs25, Pfs25, and Pgs28 share immunochemical characteristics as well. Transmission-blocking mAbs against this family of proteins with EGF-like domains recognize nonreduced, but not reduced, parasite extracts. Further, as has been found with Pfs25 (D. C. Kaslow, unpublished data), polyclonal sera raised against reduced Pgs28 have substantially diminished transmission-blocking activity compared with that raised against nonreduced Pgs28 (Table 1). Now that the amino acid sequence for Pgs28 has been deduced, it is clear that the target epitopes of transmission-blocking antibodies in this family of sexual stage antigens depend on proper disulfide bonding within EGF-like domains.

In addition, in this family of proteins, polyclonal monospecific antisera are qualitatively, and not just quantitatively, superior to mAbs in blocking infectivity (12). The superiority of polyclonal sera could be accounted for by a mixture of antibodies whose greater affinity and/or particular isotype render them more effective at blocking transmission. Our results, however, suggest an alternative or at least complementary explanation. Polyclonal antisera impair more than one stage of parasite development, possibly by acting against more than one epitope, some of which may confer stage-specific susceptibility to immune intervention. It appears that polyclonal sera combine these stage-specific effects to provide vastly improved transmission-blocking activity. Whether combining these antigens in a single cocktail vaccine will also markedly enhance and prolong transmission-blocking activity can now be addressed.

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Address correspondence to David C. Kaslow, Molecular Vaccine Section, Laboratory of Malaria Research, NIAID, Building 4 Room B1-37, National Institutes of Health, Bethesda, MD 20892.

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